Studies on the Biosynthesis of Lincomycin. II. Antibiotic U-11,973, *N*-Demethyl Lincomycin*

A. D. Argoudelis, J. A. Fox, and D. J. Mason

ABSTRACT: U-11,973 is a new antibiotic produced by *Streptomyces lincolnensis* var. *lincolnensis* when α-methylthiolincosaminide is added in the fermentation media. α-Methylthiolincosaminide, which is obtained by hydrazinolysis of lincomycin, is one of the two moieties present in the lincomycin molecule. U-11,973 and lincomycin, produced under the conditions of the fermentation, are recovered from the fermentation beer by adsorption on carbon followed by elution with aqueous acetone.

Separation of U-11,973 and lincomycin was achieved by solvent extraction and fractional crystallization of their hydrochloride salts. Nuclear magnetic resonance studies and degradative and synthetic work establish the structure of antibiotic U-11,973 as being *N*-demethyl lincomycin.

The isolation of lincomycin,[1] a new antibiotic produced by *Streptomyces lincolnensis* var. *lincolnensis*, has been reported by Herr and Bergy (1962). Chemical studies carried out by Hoeksema and his co-workers resulted in the elucidation of the complete structure of lincomycin (compound Ia) including the stereochemistry at all asymmetric centers (Hoeksema *et al.*, 1964). Argoudelis *et al.* (1965) have recently described the isolation of antibiotic U-21,699 (compound Ib) which differs from lincomycin in the hygric acid portion of the molecule. On the other hand, Argoudelis and Mason (1965) isolated antibiotic U-11,921 (compound Ic) which is produced by *S. lincolnensis* when DL-ethionine is added to the fermentation medium. Structural studies showed that the $-SCH_3$ group, which is present in lincomycin, has been substituted by an $-SCH_2CH_3$ group in U-11,921.

The first step in the degradation of lincomycin is the cleavage of the antibiotic by hydrazine to α-methylthiolincosaminide (compound II) and the hydrazide of 4-*n*-propyl-L-hygric acid (compound IIIa). Acid hydrolysis of the hydrazide afforded crystalline 4-*n*-propyl-L-hygric acid (compound IVa) (Hoeksema *et al.*, 1964). As part of the studies on the biosynthesis of lincomycin, we decided to determine what effect the addition of 4-*n*-propyl-L-hygric acid and α-methylthiolincosaminide would have on the fermentation of *S. lincolnensis*. Although no increase in lincomycin production was observed after adding either moiety, the addition of α-methylthiolincosaminide resulted in the production of a new biologically active compound designated U-11,973, which was found to be *N*-demethyl lincomycin by the studies described in the next section. The significance of the formation of U-11,973 is not known at this time. Biological properties of antibiotic U-11,973 have been described by Mason and Lewis (1964).

Experimental and Results

Fermentation Procedures. Seed cultures of *S. lincolnensis* var. *lincolnensis* were prepared in a medium consisting of glucose monohydrate (Cerelose), 10 g/liter; *N*-Z-Amine B, 5 g/liter; and Yeastolac, 10 g/liter. The cultures were incubated at 28° for 48 hours on a rotary shaker. A fermentation medium consisting of glucose monohydrate (Cerelose), 15 g/liter; starch, 40 g/liter; blackstrap molasses, 20 g/liter; Pharmamedia, 25 g/liter; and $CaCO_3$, 8 g/liter, was inoculated at a rate of 5% (v/v) with the 48-hour seed medium. The fermentations were incubated at 28° on a rotary shaker (250 rpm, 6-cm stroke). Methylthiolincosaminide was added to fermentations of *S. lincolnensis* var. *lincolnensis* at levels of 0.5–4 g/liter on day 0, 1, 2, or 3 of the fermentation. Best results were obtained when 2 g/liter of methylthiolincosaminide was added on the second day (48 hours after inoculation). Fermentation beers were normally harvested after 120 hours. Under the conditions used, both lincomycin and U-11,973 were produced. The production of U-11,973 was followed by paper and thin-layer chromatography. The paper chromatogram pattern and a typical thin-layer chromatogram are presented in Figures 1 and 2, respectively.

Isolation of U-11,973. Recovery from the Fermentation Broth. Fermentation broth (35 liter) was filtered at harvest *p*H using filter aid. The mycelial cake was washed with water and the cake was discarded. The filtrate was stirred for 30 minutes with activated carbon (5%, w/v). The mixture was filtered and the filtrate was discarded. The carbon cake was washed successively with water and 20% aqueous acetone, then eluted

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[1] Lincocin is the trademark of The Upjohn Company for lincomycin hydrochloride.

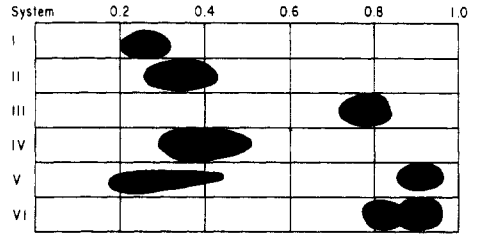


FIGURE 1: Paper chromatography of U-11,973. Solvent systems: (I) 1-butanol-water (84:16), developed 16 hours; (II) 1-butanol-water (84:16) plus 0.25% *p*-toluenesulfonic acid, developed 16 hours; (III) 1-butanol–acetic acid–water (2:1:1), developed 16 hours; (IV) 1-butanol-water (84:16) plus 2% piperidine, developed 16 hours; (V) 1-butanol-water (4:96), developed 5 hours; (VI) 1-butanol-water (4:96) plus 0.25% *p*-toluenesulfonic acid, developed 5 hours. The antibiotic was detected by bioautography of *Sarcina lutea* seeded agar.

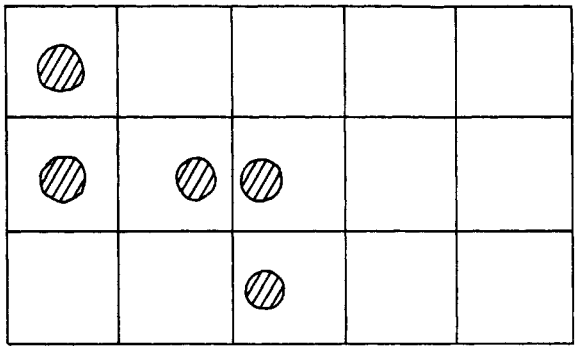


FIGURE 2: Thin-layer chromatography of U-11,973. Upper, U-11,973; middle, U-11,973, U-21,699, and lincomycin; lower, lincomycin. Thin-layer plates were prepared from Silica Gel G (Merck Darmstadt). Thickness of the film was 0.4 mm. The solvent system consisted of 150 ml of methyl ethyl ketone, 50 ml of acetone, and 20 ml of water. Detection systems used: periodate permanganate spray, and bioautography on *Sarcina lutea* seeded agar.

once with 70% aqueous acetone and twice with 90% aqueous acetone. The acetone eluates were combined and concentrated to an aqueous solution which was freeze-dried to give 121.7 g of a crude mixture of lincomycin and U-11,973 (prepn A). The carbon cake was then slurried with 1-butanol-acetone and water mixture in the volume ratio of 3:3:2. The mixture was filtered and the filtrate was concentrated to an aqueous solution which was freeze-dried to give 9.0 g of colorless material which was found to be a mixture of lincomycin and U-11,973 (prepn B). Preparations A and B were combined and dissolved in 400 ml of water. The *p*H of the solution was adjusted to 10 with aqueous sodium hydroxide solution. The alkaline solution was then extracted successively, once with 500 ml of Skellysolve B, and four times with 250-ml portions of methylene chloride. The Skellysolve extract was discarded. The methylene chloride extracts were concentrated to dryness to give 4.5 g of amorphous material which was found, by thin-layer chromatography, to contain lincomycin only. The spent aqueous solution was then extracted five times with 250-ml portions of 1-butanol. The combined butanol extract was concentrated to dryness. The residue was dissolved in 50 ml of 0.5 N methanolic hydrogen chloride and this solution was mixed with 500 ml of ethyl ether. The precipitated material was isolated by filtration. This material containing U-11,973 and lincomycin as the hydrochloride salts was used as the starting material for the countercurrent distribution described below.

Countercurrent Distribution. Isolation of Crystalline U-11,973 Hydrochloride. Crude U-11,973 hydrochloride obtained as described was dissolved in 100 ml of the lower phase of the solvent system consisting of equal volumes of 1-butanol and water. This solution was mixed with equal volumes of the upper phase of the foregoing solvent system and transferred to an all-glass, Craig countercurrent distribution apparatus. After 930 transfers, the distribution was analyzed by solids determination and thin-layer chromatography. Tubes 104–170, which contained mainly U-11,973 hydrochloride, were combined (660 ml) and concentrated to a volume of 80 ml. Crystalline U-11,973 hydrochloride formed, was isolated by filtration and dried (200 mg). An additional 200 mg was obtained by further concentration of the filtrate. These two preparations were combined and dissolved in water (17 ml). Addition of 60 ml of acetone resulted in the crystallization of U-11,-973 hydrochloride in the form of colorless, long, feathery crystals, which were isolated by filtration and dried; yield 170 mg, $[\alpha]_D^{25}$ +149° (*c* 0.9, water); the infrared spectrum of U-11,973 hydrochloride in Nujol (Figure 3) shows absorption bands in the OH, NH (3478, 3305, 3220 cm^{-1}), and in the carbonyl region (1687, 1668 cm^{-1}). Other absorption bands are present at 1596, 1350, 1308, 1277, 1240, and 1201 cm^{-1}.

Anal. Calcd for $C_{17}H_{32}N_2O_6S \cdot HCl \cdot H_2O$: C, 45.73; H, 7.90; N, 6.28; S, 7.18; Cl, 7.94; O, 25.08; mw, 446.5. Found: C, 45.62; H, 7.78; N, 6.23; S, 7.31; Cl, 7.82; O (by difference), 25.24.

Potentiometric titration showed the presence of one basic group, pK_a' 7.60; equivalent weight found, 445, 450.

Transformation of U-11,973 to Lincomycin. U-11,973 hydrochloride (600 mg) was dissolved in 100 ml of water. The *p*H was adjusted to 9.7 using Dowex 2 anion exchange resin in the hydroxide form. The alkaline solution was then freeze-dried to give U-11,973. This material was dissolved in 50 ml of methylene chloride and 5 ml of absolute methanol. The solution was mixed with 2.4 ml of methyl iodide and the mixture was allowed to stand at room temperature for 1.5 hours. It was then concentrated to dryness. The residue was dissolved in 100 ml of water, and the *p*H of the solution was ad-

justed to 9.6 with Dowex 2 in the hydroxide form. The alkaline solution was extracted three times with 70-ml portions of chloroform. The chloroform extract was concentrated to dryness to give 200 mg of colorless amorphous material which was found, by thin-layer chromatography, to be lincomycin. The spent aqueous was freeze-dried to give 390 mg, found to be U-11,973. Further purification of the obtained lincomycin was achieved by Florisil chromatography described in the following paragraph.

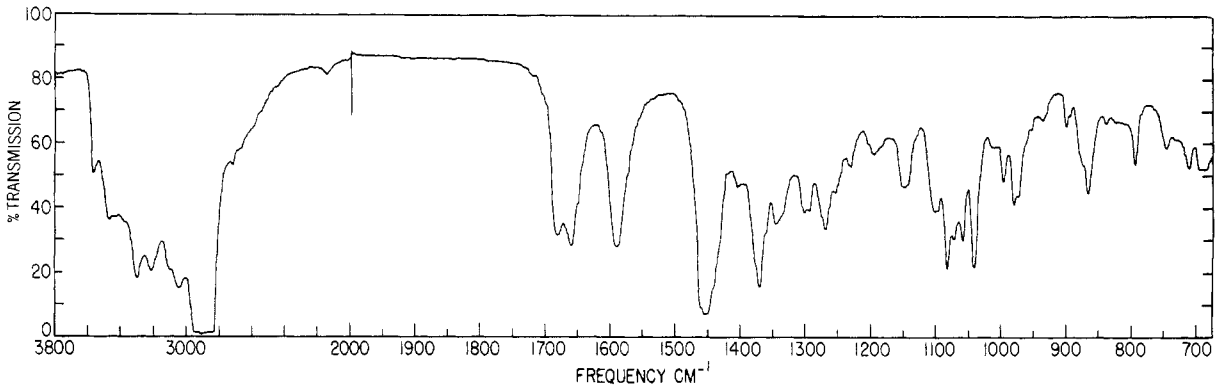


FIGURE 3: Infrared spectrum of U-11,973 (in mineral oil suspension).

Florisil Chromatography of Lincomycin. The column was prepared by pouring 10 g of Florisil in a column containing Skellysolve B, and allowing the adsorbent to settle under atmospheric pressure. The crude lincomycin obtained above was dissolved in small amounts of methylene chloride and the solution was added on top of the Florisil bed. The column was then eluted with Skellysolve B–acetone mixtures of increasing acetone content. Lincomycin was eluted with a Skellysolve B–acetone mixture in the volume ratio of 40:60. Fractions containing lincomycin were combined, and the solution was mixed with 0.8 ml of 1 N methanolic hydrogen chloride. The precipitated crystalline material was isolated by filtration and dried; yield 65 mg. This material was identified as being lincomycin hydrochloride by paper and thin-layer chromatography, specific rotation, and by comparison of NMR[2] and infrared spectra to those of an authentic sample of lincomycin.

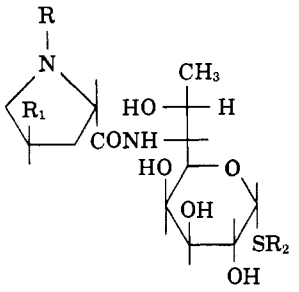
Hydrazinolysis of U-11,973. Isolation of α-Methylthiolincosaminide. One g of U-11,973 was treated with 25 ml of hydrazine hydrate according to the procedure described by Hoeksema and his co-workers (Hoeksema *et al.*, 1964). Material insoluble in acetonitrile was recrystallized from dimethylformamide to give 350 mg of a colorless crystalline compound which was identified as α-methylthiolincosaminide by comparison of specific rotation and infrared and NMR spectra with those of an authentic sample obtained from lincomycin.

Acid Hydrolysis. Isolation of 4-Propyl-L-proline Hydrochloride. The filtrate obtained from the trituration of the hydrazinolysis mixture with acetonitrile (Hoeksema *et al.*, 1964) was concentrated to dryness. The residue was kept at reflux with 20 ml of 6 N aqueous hydrochloric acid for 6 hours. The solution was then concentrated to dryness. The residue was dissolved in 30 ml of water, and the *p*H was adjusted to 6.5 with solid silver carbonate. Insoluble material was removed by filtration, and the filtrate was decolorized with carbon. The mixture was then filtered and the clear filtrate was concentrated to dryness. The residue was dissolved in 1 ml of 1 N methanolic hydrogen chloride and 2 ml of ethanol. Addition of ether afforded crystalline 4-propyl-L-proline hydrochloride; yield 26 mg.

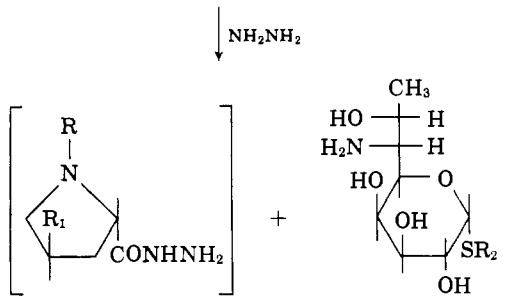
Discussion

U-11,973 has been isolated and characterized as the crystalline hydrochloride salt. Analytical values suggested the molecular formula, $C_{17}H_{32}N_2O_6S\cdot HCl\cdot H_2O$; mw 446.5. Potentiometric titration showed the presence of one basic group with pK_a' of 7.60 (60% aqueous ethanol); equivalent weight 445, 450. The specific rotation of U-11,973 was found to be $[\alpha]_D^{25}$ +149° (*c* 0.9, water). The infrared spectrum (Figure 3) shows absorption bands in the —OH, —NH region and also at 1687, 1596 cm^{-1} indicating the presence of an amide linkage. The above-mentioned properties of U-11,973 hydrochloride resemble those of lincomycin and especially those of antibiotic U-21,699 (Argoudelis *et al.*, 1965). However, U-11,973 could be easily differentiated from both antibiotics by thin-layer chromatography (Figure 2). U-11,973 hydrochloride showed solubilities considerably different from those of the other lincomycin-related antibiotics. For example, its solubility in water is about one-tenth of that of lincomycin. Because of the limited solubility of U-11,973 in solvents commonly used for NMR, the spectrum obtained gave lim-

[2] Abbreviation used in this work: NMR, nuclear magnetic resonance.

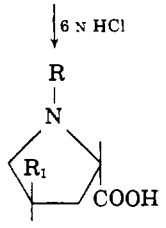


Ia, $R=CH_3$; $R_1=CH_2CH_2CH_3$; $R_2=CH_3$
Ib, $R=CH_3$; $R_1=CH_2CH_3$; $R_2=CH_3$
Ic, $R=CH_3$; $R_1=CH_2CH_2CH_3$; $R_2=CH_2CH_3$
Id, $R=H$; $R_1=CH_2CH_2CH_3$; $R_2=CH_3$



IIIa, $R=CH_3$; $R_1=CH_2CH_2CH_3$
IIIb, $R=H$; $R_1=CH_2CH_2CH_3$

II, $R_2=CH_3$



IVa, $R=CH_3$; $R_1=CH_2CH_2CH_3$
IVb, $R=H$; $R_1=CH_2CH_2CH_3$

ited information regarding its structure. Comparison of the NMR spectra[3] of lincomycin and U-11,973 showed that both compounds have similar absorption peaks in the area of 0–150 cps, with the only exception being that the peak attributable to the $-NCH_3$ group present in lincomycin is missing in the spectrum of U-11,973. Specifically, the NMR spectrum suggested the presence of the following groups or structural features in the U-11,973 molecule: (1) $CH_3CH_2CH_2-$, (2) CH_3S-, (3) $CH_3CH(O-)-$, (4) hygric acid or proline nucleus, and (5) anomeric hydrogen.

Combination of the information derived from NMR spectra with analytical, infrared, and specific rotation data suggested structure Id as the structure of U-11,973. Complete proof of this structure was obtained by reacting U-11,973 with methyl iodide and isolating lincomycin hydrochloride (compound Ia), identified as such by paper and thin-layer chromatography, specific rotation, and infrared and NMR spectra. In addition, hydrazinolysis of U-11,973 followed by acid hydrolysis afforded α-methylthiolincosaminide (compound II), identical with an authentic sample obtained from lincomycin, and 4-*n*-propyl-L-proline (compound IIIb) isolated as the crystalline hydrochloride salt.

U-11,973 has been used as the starting material for a series of *N*-alkyl analogs of lincomycin which have shown interesting biological properties. The chemistry of these compounds will be the subject of a future communication.

Acknowledgments

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[3] Spectra were calibrated in cps units at 60 Mc, downfield from internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate. Spectra were observed with a Varian A-60 spectrometer on solutions (*ca.* 0.4 ml, *ca.* 0.25 M) of the compounds in deuterium oxide. The helpful discussions with Messrs F. A. MacKellar and J. F. Zieserl of The Upjohn Company are gratefully acknowledged.